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Research Note

Campylobacter coli Naturally Resistant to Elevated Levels of Gentamicin as a Marker Strain in Poultry Research†

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ABSTRACT

Campylobacter inoculation studies are limited without a suitable marker strain. The purpose of this study was to screen *Campylobacter* strains (n = 2,073) obtained from poultry carcass rinses through the Centers for Disease Control and Prevention's National Antimicrobial Resistant Monitoring System for resistance to gentamicin and evaluate one strain's efficacy as a marker. A *C. coli* strain was found resistant to gentamicin at >32 μg/ml. Gentamicin was incorporated into media (Campy-Cefex agar, *Brucella* agar, and blood agar) from 0 to 1,000 μg/ml, and the upper level of gentamicin resistance was determined. *C. coli* strain's upper level of growth on Campy-Cefex plates, blood agar plates, and *Brucella* agar plates was 400, 300, and 200 μg/ml, respectively. Ceca and postpick carcass rinses were obtained and streaked onto Campy-Cefex agar at the above gentamicin levels to evaluate background microflora exclusion. Campy-Cefex agar containing gentamicin at 100 μg/ml prevented from the ceca, and reduced from the rinse, background microflora. The *C. coli* strain was orally or intracloacally inoculated into chicks. At 1, 3, and 6 weeks of age, inoculated broilers were removed and several tissue types sampled for the presence of the marker strain. At 6 weeks of age, 10 additional noninoculated penmates were sampled. The *C. coli* strain colonized chicks, disseminated to body tissues, colonized penmates, and persisted throughout the 6-week grow-out. The *C. coli* strain's unique characteristic, being resistant to high levels of gentamicin, allows for a marker that can be used in a wide range of *Campylobacter* research projects.

The Centers for Disease Control and Prevention estimates that in the United States, human campylobacteriosis accounts for more than 2.4 million cases of gastroenteritis annually, and 80% of the cases are considered foodborne *(18, 32)*. The two major *Campylobacter* spp. associated with illness are *C. jejuni* and *C. coli*. *Campylobacter* spp. are commonly carried in the alimentary tract of poultry in levels up to 10^8 to 10^9 CFU/g of luminal content and can be isolated most frequently from the ceca of poultry *(21)*. The *Campylobacter* prevalence for broilers within a contaminated flock entering the processing plant can reach 100% *(41)*. The average prevalence of infected broiler flocks is typically 44 to 59% *(24, 36)*. The number of contaminated broilers accounts for the high incidence of *Campylobacter* spp. in poultry processing plants and on processed carcasses *(2, 25)*. The average *Campylobacter* spp. prevalence on chicken carcasses and meat at retail is 57%, with a range of 23 to 100% *(24)*. The high colonization prevalence in poultry and the resultant clinical infection of humans have prompted investigations focused on identifying and subsequently eliminating sources of *Campylobacter* contamination within poultry flocks *(8, 31)*.

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Developing and using suitable *Campylobacter* strains in research projects as markers could allow for cocolonization studies to be conducted that are currently not feasible or very difficult to conduct. Performing inoculation studies with characterized strains of *Campylobacter* presently available may require utilization of molecular techniques to determine the similarity of the isolates obtained to the isolates used for inoculation in the study. Marker or surrogate organisms have been used extensively in research studies *(16, 23, 26)*. In particular, marker organisms with a unique antibiotic resistance pattern have been very beneficial when conducting controlled inoculation studies *(3, 11)*. Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic and is either chromosomal or plasmid mediated. Chromosomal resistance usually evolves naturally or by a stressor on a population. When antibiotic resistant genes are generated, this information can then be transferred horizontally between bacterial isolates by chromosomal loci or plasmid exchange *(2)*. This ability has been used by numerous researchers in order to develop antibiotic-resistant organisms through transformation protocols.

Campylobacter strains can exhibit resistance to a range of antibiotics *(33)*. A few of the more common classes of antibiotics that *Campylobacter* strains are tested against include aminoglycosides, macrolides, quinolones, and tetracyclines *(35)*. *Campylobacter* strains are generally more re-

sistant to tetracyclines, quinolones, and macrolides than they are to aminoglycosides *(1, 20, 22, 33)*. Gentamicin is an aminoglycoside that is commonly used as an antibiotic in food-animal productions and human medicine, and *Campylobacter* strains generally are susceptibility to gentamicin *(20, 22)*. A *Campylobacter* strain highly resistant to gentamicin could serve as a marker organism for controlled research challenge studies. The objectives of this study were (i) to screen *Campylobacter* strains obtained from poultry carcass rinses for a unique gentamicin-resistant pattern, (ii) to determine level of gentamicin resistance, (iii) to determine whether gentamicin incorporated into media could exclude background microflora, and (iv) to determine whether the isolate could colonize and disseminate in broilers, similar to other natural *Campylobacter* strains.

MATERIALS AND METHODS

Antibiotic resistance marker strain identification. The antibiotic resistance patterns of 2,073 *Campylobacter* isolates obtained over 4 years (2002 to 2005) from processed poultry carcass rinses as part of the Centers for Disease Control and Prevention, National Antimicrobial Resistant Monitoring System (NARMS) were evaluated for a unique antibiotic resistant pattern to gentamicin. NARMS methods are described in detail elsewhere *(9)*.

Gentamicin resistance level. In the NARMS system, resistance up to 32 μg/ml is the maximum tested; therefore, to determine the upper level of resistance of the strain to gentamicin, two replications were performed in which gentamicin (Sigma, St. Louis, MO) was incorporated into Campy-Cefex agar, *Brucella* agar, and blood agar (Acumedia Manufacturers, Inc., Baltimore, MD) at 0, 50, 100, 200, 300, 400, 600, 800, and 1,000 μg/ml. For both repetitions, the *C. coli* isolate was streaked onto five agar plates of each type of medium, which contained the varying levels of gentamicin, and onto plates that did not contain the antibiotic. The agar plates were incubated in a microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N) at 42°C for 48 h. The plates were then evaluated for the presence of the marker *C. coli* strain.

Inhibition of natural *Campylobacter* and extraneous microflora on Campy-Cefex plates. To evaluate the ability of incorporating gentamicin into Campy-Cefex agar to inhibit natural *Campylobacter* and suppress extraneous microflora, commercial broilers and defeathered broiler carcass rinsates were used. Six-week-old broilers ($n = 5$) were acquired from a commercial facility, euthanized, and the ceca aseptically removed. The ceca were placed in a sterile sampling bag on ice and transported back to the laboratory. The ceca samples in bags were macerated with a rubber mallet, and 3× 1% (wt/vol) phosphate buffer added. The ceca samples were then stomached for 30 s. Carcasses ($n = 5$) were obtained after defeathering from a commercial processing plant, transported back to the laboratory, and carcass rinsate was obtained *(15)*. A 0.1-ml aliquot from ceca and rinse samples was streaked onto Campy-Cefex agar plates. A 0.1-ml aliquot was also streaked onto Campy-Cefex agar plates containing 100, 200, 300, 400, 600, 800, and 1,000 μg/ml gentamicin. All plates were incubated in a microaerobic atmosphere at 42°C for 48 h. After incubation, plates were observed for presumptive *Campylobacter* colonies. Suspect colonies were confirmed by microscopic observation of characteristic cellular morphology and motility in wet-mount preparations, and further confirmed through latex agglutination (PANBIO, Inc., Columbia, MD).

Principles of the so-called ecometric technique were used to measure background microflora *(27, 34)*. The method involves streaking aliquots from the carcass rinses into quadrants on the plating media. The initial aliquot (0.1 ml) was streaked onto the plating media and designated quadrant 1. Then, from that quadrant, a 0.1-ml aliquot was streaked into another quadrant and designated quadrant 2. This was repeated for designated quadrants 3 and 4. When streaking from the first to the fourth quadrant, a sterile loop was used between plating media quadrants. After incubation, the growth of non-*Campylobacter* colonies on Campy-Cefex was expressed as the absolute growth index. Growth on all four quadrants (1 to 4) was nominated an absolute growth index of 4; growth on quadrants 1 to 3 was an absolute growth index of 3, and so on. A larger quadrant number indicates a greater breakthrough of microflora.

Colonization and dissemination potential in broilers. Day-of-hatch chicks were administered a 0.1-ml suspension of 5.2 log of the marker *C. coli* strain by oral gavage ($n = 9$) or intracloacal inoculation ($n = 9$) and wing banded. The orally inoculated chicks were placed in a pen (39.6 m^2) containing 41 penmates in one experimental room, and the intracloacally inoculated chicks were placed in an identical pen located in a separate room. All chicks were placed on clean pine shavings and raised in an environmentally controlled house. At 1 week of age, inoculated broilers ($n = 3$) from each room were removed, euthanized, and the thymus, spleen, liver-gallbladder, unabsorbed yolk, bursa, and ceca aseptically removed and placed into individual sterile sampling bags. The samples were placed on ice and transported back to the laboratory. At 3 weeks of age, inoculated broilers ($n = 3$) from each room were removed, and the above sites sampled except for the bursa. At 6 weeks of age, the remaining inoculated broilers were removed, and the above samples taken. In addition, at 6 weeks of age, the ceca from noninoculated penmates ($n = 10$) were also sampled. Stepped-on drag swabs ($n = 2$ per pen) were collected weekly within each pen to evaluate environmental contamination *(5)*.

The thymus, spleen, liver-gallbladder, unabsorbed yolk, bursa, and ceca samples were macerated with a rubber mallet and 3× (wt/vol) Bolton's enrichment broth (containing 5% lysed horse blood cells) with supplements added. For the stepped-on drag swabs, 100 ml of Bolton's enrichment broth (containing 5% lysed horse blood) with supplements was added. The samples were stomached for 30 s, and 0.1-ml aliquots direct plated onto Campy-Cefex plates containing 200 μg/ml gentamicin. The agar plates were incubated in a microaerobic atmosphere (5% O_2, 10% CO_2, and 85% N) at 42°C for 48 h. The samples containing the enrichment were incubated in a microaerobic atmosphere at 42°C for 48 h. After incubation, a 0.1-ml aliquot was plated, and the above procedures for direct plating were followed. Presumptive colonies were confirmed by microscopic observation of characteristic cellular morphology and motility in wet-mount preparations and further confirmed through latex agglutination (PANBIO, Inc.).

Statistical analysis. Overall incidence of *Campylobacter* resistant to gentamicin is expressed as the percentage of all strains evaluated. Data on the level of gentamicin resistance of the one *Campylobacter* strain are expressed as either growth or no growth on different agars incorporating gentamicin. For extraneous microflora determination, the Tukey-Kramer multiple comparison test was used and the P value was set at <0.05. For colonization and dissemination, data are expressed as number of positive samples for the *Campylobacter* strain of the total number of samples.

RESULTS AND DISCUSSION

A resistance to gentamicin >32 μg/ml was observed in 1 (0.0005%) of 2,073 *Campylobacter* strains evaluated.

TABLE 1. *Antimicrobial resistance characteristics of the* Campylobacter coli *strain recovered from poultry carcass rinses in the United States in 2003*

Antimicrobial	MIC (μg/ml)
Azithromycin	0.12
Ciprofloxacin	0.06
Clindamycin	0.25
Erythromycin	1
Florfenicol	1
Gentamicin	>32
Nalidixic acid	8
Telithromycin	1
Tetracycline	>64

FIGURE 1. *Pulsed-field gel electrophoresis banding pattern of the gentamicin-resistant* Campylobacter coli *by using* SmaI.

The antibiotic resistance profile of the *C. coli* strain is provided in Table 1, and the pulsed-field gel electrophoresis pattern is shown in Figure 1. The *C. coli* strain was found to be naturally resistant to 400 μg/ml gentamicin, using Campy-Cefex agar (Table 2). A *Campylobacter* strain with this level of resistance to gentamicin is extremely rare. An internationally accepted criterion for the susceptibility testing of *Campylobacter* is not established, and the resistance breakpoint for gentamicin varies between studies *(33).* A wide variation in breakpoints makes it difficult to evaluate *Campylobacter* antimicrobial resistance *(19).* Regardless of the ranges used for gentamicin resistance, generally at levels >8 μg/ml, a very low percentage of *Campylobacter* strains are resistant *(20, 22, 33).* However, a high level (22%) of *Campylobacter* isolates resistant to gentamicin at >10 μg/ml was reported in animals and foods in Spain *(39).* In a survey from 1997 to 2001 in the United States, 1 of 1,336 *Campylobacter* isolates evaluated were gentamicin resistant and the strain was *C. coli (22).* A 2003 survey of 378 *Campylobacter* isolates from retail raw meats found no gentamicin-resistant strains *(20). Campylobacter* isolates from 342 broiler flocks were evaluated, and all isolates were susceptible to gentamicin *(43).* Four hundred forty-eight *Campylobacter* isolates were evaluated from feedlot cattle, and only a single *C. jejuni* was resistant to gentamicin *(17).*

The *C. coli* marker's ability to maintain this high level of gentamicin resistance is still unknown. However, the strain's resistance to gentamicin appears to be stable. The culture was first recovered in 2003. Since then, the culture has been stored at −80°C and has been removed from the ultralow-temperature freezer (more than 10 times) and transferred onto plating media numerous times over the past year for research purposes, without losing resistance to gentamicin. The strain has been used by other researchers in the United States in processing studies and maintains a high level of gentamicin resistance. The mechanism for resistance in this particular strain needs to be determined. *Campylobacter* spp. contain genetic mechanisms for natural transformation and conjugation *(1).* This indicates that transfer between strains can occur. An increase in *Campylobacter* spp. MIC for gentamicin has been shown by using an efflux pump inhibitor *(29).* However, gentamicin resistance appears to be more often driven by production of the aminoglycoside-modifying enzymes, and once modification occurs, this trait seems to be stable *(28, 39, 42).* If this trait is on the plasmid or transposon of the marker *C. coli* strain, then transfer to other *Campylobacter* may occur. However, this transfer has not been observed in a *C. jejuni* strain used in coinoculation studies with the marker *C. coli* strain (unpublished data).

Gentamicin is an antibiotic in the aminoglycoside group and is active against many gram-negative organisms and some gram-positive organisms *(42).* Gentamicin is bactericidal by targeting accessible regions of polyanionic 16S rRNA on the 30S ribosome *(7, 42).* The antibiotic is heat stable and can remain active after autoclaving, which has advantages when preparing media. Incorporating gentamicin into the Campy-Cefex medium was significantly ($P = 0.01$) beneficial in controlling breakthrough of extraneous microflora from ceca samples, compared with regular Campy-Cefex medium. From ceca samples, the number of quadrants containing background microflora averaged 2.6 on regular Campy-Cefex plates, and background microflora was not present on any of the quadrants on Campy-Cefex plates with gentamicin incorporated from 100 to 1,000 μg/ml. From rinse samples, the number of quadrants containing microflora averaged 2.2 on regular Campy-Cefex, and 1.2, 1.1, 1.3, and 0.4 on Campy-Cefex plates with gentamicin incorporated at 100, 200, 300, and 400 μg/ml, respectively. No background microflora was observed at levels >400 μg/ml. A significant ($P < 0.001$) difference be-

TABLE 2. *Ability of a marker* Campylobacter coli *to grow on Campy-Cefex agar,* Brucella *agar, and blood agar plates with varying levels of gentamicin*[a]

	Gentamicin level (μg/ml):								
Agar	0	50	100	200	300	400	600	800	1,000
Campy-Cefex	G (10/10)	G (10/10)	G (10/10)	G (10/10)	G (10/10)	G (10/10)	NG (0/10)	NG (0/10)	NG (0/10)
Brucella	G (10/10)	G (10/10)	G (10/10)	G (10/10)	NG (0/10)	NG (0/10)	NG (0/10)	NG (0/10)	NG (0/10)
Blood	G (10/10)	G (10/10)	G (10/10)	G (10/10)	G (10/10)	NG (0/10)	NG (0/10)	NG (0/10)	NG (0/10)

[a] G, growth of *C. coli* strain on plates; N, no growth of *C. coli* strain on plates. Number of plates positive for *C. coli*/number of plates streaked are presented in parentheses.

TABLE 3. *Evaluation of the* Campylobacter coli *strain colonization and dissemination ability in broilers after oral or intracloacal inoculation of day-of-age birds*[a]

Week sampled	Sample site: Thymus		Spleen		Liver-gallbladder		Unabsorbed yolk sac		Bursa		Ceca	
	OR	IC	OR	IC	OR	IC	OR	IC	OR	IC	OR	IC
1	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	3/3	3/3	3/3	3/3
3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	NS	NS	0/3	0/3
6	0/3	1/2	0/3	1/2	1/3	1/2	0/3	1/2	NS	NS	3/3	1/2
6 (PM)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	10/10	8/10

[a] Values are number of plates positive/number sampled. OR, oral gavage route of inoculation; IC, intracloacal route of inoculation; NS, not sampled; PM, penmates of inoculated birds.

tween Campy-Cefex and Campy-Cefex with gentamicin was observed when incorporating levels of gentamicin into the media from 400 to 1,000 μg/ml. All ceca and carcass rinse samples contained natural *Campylobacter,* but they did not grow on Campy-Cefex plates in which gentamicin was incorporated.

The *C. coli* gentamicin-resistant isolate was found to colonize chicks by either route of inoculation, disseminate to body tissues, result in the colonization of noninoculated penmates, and persist throughout a 6-week broiler grow-out (Table 3). The *C. coli* strain was recovered from the litter in each pen weekly throughout the grow-out by using stepped-on drag swabs. Interestingly, at 3 weeks' postinoculation, the *C. coli* strain was not recovered from birds that had been inoculated, but was recovered from inoculated birds at 1 and 6 weeks' postinoculation. The absence of colonization in inoculated birds over time has been observed in other studies *(38).* The reason for the lack of colonization in these six birds is not fully understood, since the marker was recovered from the litter at each weekly sampling. If this was due to the colonization factor of the marker strain, then this should have also been observed at other sampling intervals; therefore, the findings could be related to experimental variation. Presence of maternal antibodies may have limited colonization in these birds *(40).* The microbial diversity of the intestinal bacterial community of the maturing broilers could have also contributed to noncolonization *(30).*

Inoculated *C. jejuni* has been shown to disseminate to internal tissues and organs, regardless of inoculation route for at least 1 week postinoculation *(10).* In the present study, the *C. coli* strain disseminated to internal tissues and organs of inoculated birds at 1 and 6 week(s) postinoculation. *Campylobacter* spp. are recovered from tissues and organs throughout the bird's body in commercial settings *(10, 12, 13). C. jejuni and C. coli* are present in the spleen, liver-gallbladder, and unabsorbed yolks of commercial broilers at market age *(12, 13).* Continual dissemination and presence in these sites on a weekly basis throughout a grow-out is unknown.

In order for adequate development of intervention and reduction in *Campylobacter* spp. colonization rate of broiler flocks, an improved understanding of the ecology of *Campylobacter* spp. in poultry is needed. The source of broiler flock infections, modes of transmission, and environmental factors that contribute to the spread of *Campylobacter* spp. on poultry farms are beginning to be determined *(4, 6, 15).* This strain allows inoculation studies to be conducted with commercial birds, processed carcasses, or in other settings where natural *Campylobacter* spp. are present. In a separate efficacy study, the *C. coli* strain was an effective marker organism when studying the effects of chlorine or chlorine dioxide during immersion chilling of commercial broilers on *Campylobacter (37).* In the current study, resistance to gentamicin at 400 μg/ml allowed for evaluation of the *C. coli* strain as a potential *Campylobacter* marker organism. The *C. coli* strain was efficacious in inoculation studies within broilers and could be used for numerous future inoculation or poultry research studies particularly involving cocolonization.

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